Association of neprilysin polymorphism with cerebral amyloid angiopathy

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Objectives: The risk of sporadic cerebral amyloid angiopathy (CAA) may be associated with genetic polymorphisms of molecules related to anabolism or catabolism of amyloid β protein (Aβ). The authors investigated whether a polymorphism of the gene (*NEP*) coding for neprilysin, an enzyme catabolising Aβ, is associated with CAA.
Methods: The study analysed the GT repeat polymorphism in the enhancer/promoter region of *NEP* and severity of CAA in 164 necropsied elderly Japanese subjects.
Results: The subjects had *NEP* polymorphisms with 19 to 23 GT repeats and were classified into nine genotypes. CAA severity was significantly higher in the subjects with up to 40 repeats in total than those with more than 40 repeats (p=0.005). There was a significant correlation between the number of the shorter alleles (19 or 20 repeats) and CAA severity (p=0.024). In addition, there was no interaction between the *NEP* polymorphism and apolipoprotein E genotype.
Conclusions: These results suggest the association between the *NEP* polymorphism and the risk of CAA. Further study using more samples from populations with different ethnic backgrounds is necessary.

C erebral amyloid angiopathy (CAA) is a cerebrovascular amyloid deposition and causes intracerebral haemorrhage and other cerebrovascular disorders (see reviews[1][2]). Several types of CAA have been identified in association with various amyloid proteins including amyloid β protein (Aβ), cystatin C, prion protein, transthyretin, gelsolin, and Abri/ADan.[2] Sporadic CAA of Aβ type is most common in elderly people as well as patients with Alzheimer's disease (AD)[2]; polymorphisms in apolipoprotein E (*APOE*) and other genes[3–7] may be associated with CAA or CAA related haemorrhage.

Although the pathomechanism underlying cerebrovascular amyloid deposition remains unclear, recent studies have shown that a neuronal source of Aβ is sufficient to induce cerebrovascular amyloid deposition.[8] Aβ in the brain extracellular fluid may be internalised by cerebrovascular smooth muscle cells, leading to vascular Aβ deposition[9][10]; a major molecular species of cerebrovascular Aβ is the 40-amino acid Aβ (Aβ40), although Aβ42 is initially deposited in the vessel wall.[11]

Neprilysin has been shown to be a major proteolytic enzyme responsible for the catabolism of Aβ in the brain.[12–14] Aβ42 was deposited in the brain after infusion of the inhibitor, although Aβ40 was less affected.[12] The levels of Aβ40 as well as Aβ42 were significantly increased in the neprilysin deficient mice in a gene dose dependent manner.[14] The regional levels of Aβ in the neprilysin deficient mice correlated with the vulnerability to Aβ deposition in the human AD brain.[14] In addition, localisation of neprilysin in the human cerebral cortex was inversely correlated with the vulnerability to Aβ deposition[15]; neprilysin was reduced in high Aβ plaque areas of AD brain.[16] These findings suggest an important role of neprilysin in Aβ deposition.

Expression of the neprilysin gene (*NEP*) is transcriptionally regulated in a tissue specific manner, generating four types of neprilysin mRNA; neurons predominantly express the type 1 transcript containing exon 1.[14] [17–20] A dinucleotide GT (or TG) repeat polymorphism is present in the enhancer and promoter regions upstream of exon 1, and it may be involved in regulation of the expression level of neprilysin in neurons.[14] [17–20] Therefore, the polymorphism may influence Aβ degradation in the brain.

We previously examined the relation between the *NEP* polymorphism and AD, but failed to find any association between them.[21] As mentioned above, the major Aβ species of cerebrovascular amyloid is Aβ40, and this is in contrast with senile plaques in which Aβ42 is the most important component. Pathomechanism(s) underlying the Aβ deposition would be different between cerebrovascular and brain parenchymal tissues. Therefore, we investigated whether the *NEP* polymorphism is associated with CAA in this study. In addition, AD and *APOE* genotype were also analysed to explain the association between the *NEP* polymorphism and CAA.

METHODS
Subjects
We studied 164 Japanese patients (age, 62 to 104 years; mean (SD), 85.5 (7.8) years), from a consecutive necropsy series in a large geriatric hospital, excluding cases in which brain samples were not available for this study and cases of neurodegenerative diseases other than AD. We obtained consent from the families of all participants at necropsy. This study project was approved by the ethics committees of the institutions. They included 75 patients with AD satisfying the neuropathological criteria of the Consortium to Establish a Registry for AD (CERAD).[22] Among the 89 non-AD patients, 32 patients clinically presented with cerebrovascular disorders, including 26 patients with cerebral infarction, four with cerebral haemorrhage, and two with both infarction and haemorrhage. There was no significant difference in the age at death between AD and non-AD groups. No familial cases of AD or CAA were included. The relation between AD and *NEP* polymorphism has already been reported in these patients.[21] Some of these patients were previously studied for CAA.[6][7][23]

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Abbreviations: CAA, cerebral amyloid angiopathy; Aβ, amyloid β protein; AD, Alzheimer's disease

Table 1 Average CAA counts (mean (SE)) in the *NEP* genotypes

NEP genotype	CAA count	
19/19	19.0 (14.1)	(n=4)
19/20	31.7 (11.8)	(n=10)
19/21	38.5 (21.7)	(n=4)
20/20	20.1 (2.8)	(n=102)
20/21	6.6 (2.9)	(n=23)
20/22	0 (0)	(n=5)
20/23	0 (0)	(n=2)
21/21	18.0 (9.2)	(n=11)
21/23	3.7 (3.7)	(n=3)
Total	18.0 (2.1)	(n=164)

p=0.077 by Kruskal-Wallis test.

Neuropathological evaluation of CAA

Congophilic deposits with green birefringence under polarised light were identified as amyloid deposits. The cerebrovascular amyloid deposits were immunohistochemically confirmed to be Aβ. Using a large section of the occipital lobe (about 4×4 cm in size), the numbers of meningeal and cortical vessels with and without amyloid deposits were counted, and the percentage of amyloid laden vessels was calculated (= CAA count). We examined the occipital lobe, because it was the most frequently affected by CAA in our previous study in both AD and non-AD cases,[24] and seems suitable to detect CAA of very slight degree. The quantification was performed without knowledge of the *NEP* or *APOE* genotypes. Severe vascular wall involvement by CAA was commonly found in patients with high CAA counts.

Identification of the *NEP* polymorphism

The *NEP* polymorphism was identified as previously reported.[21] Briefly, genomic DNA, isolated from frozen brain tissue, was amplified by PCR as described by Comings *et al*.[25] GT repeats in the 5′ region of *NEP* were counted with an ALF DNA sequencer II (Pharmacia Biotech). Direct sequence analysis of PCR products of some patients with representative genotypes using ABI PRISM model 310 (Perkin-Elmer) verified the number of GT repeats. The *APOE* genotype was also determined.[21]

Statistical analyses

We compared the number of CAA counts among the *NEP* genotypes. We also compared the total repeat number and other parameters of the *NEP* polymorphism. Similar analyses were performed according to the subgroups AD or non-AD, and *APOE* ε4 status. Kruskal-Wallis, Mann-Whitney, and Spearman's rank correlation tests were used. Statistical significance was defined as $p < 0.05$. The statistical analyses were performed using StatView J-7.5 (Abacus Concepts, Berkeley, CA).

RESULTS

The subjects had *NEP* polymorphisms with 19 to 23 GT repeats and were classified into nine genotypes (table 1). The allele with 20 repeats was the most frequent (allele frequency, 0.74), followed by 21 (0.16), 19 (0.07), 22 (0.02), and 23 repeats (0.02). There was no difference in the *NEP* allele frequency between AD and non-AD cases as previously reported.[21]

Among the 164 patients, 93 presented with CAA of various degrees, and 71 had no CAA. CAA counts were not significantly different between the *NEP* genotypes in total (table 1), AD, or non-AD cases (data not shown). As shorter alleles tended to be associated with a higher CAA count (table 1), we examined the relation between the total repeat number and CAA. As shown in table 2, patients with up to 40 repeats in total had a significantly higher CAA count (21.6 (2.7)) than those with more than 40 repeats (8.2 (2.8)) (p=0.005); the difference was also significant in non-ε4 carriers, but not in AD, non-AD, or *APOE* ε4 carriers.

Furthermore, the number of the shorter (19 or 20) repeat alleles significantly correlated with CAA count in the total cases (9.9 (7.9) for no alleles; 9.0 (3.5) for one allele; 21.6 (2.7) for two alleles) (correlation coefficient = 0.176 and p=0.024 by Spearman's rank correlation test), but not in the subgroups by AD or non-AD, or in those by *APOE* ε4 carriers or non-ε4 carriers (data not shown).

In addition, the presence of *APOE* ε4 was significantly associated with AD in this population (frequency of ε4 carriers: 0.45 in AD, and 0.15 in non-AD) (p<0.0001). AD patients had a significantly higher CAA count (30.3 (3.5)) than non-AD patients (7.7 (2.1)) (p<0.0001). *APOE* ε4 carriers had a significantly higher CAA count (23.2 (3.9)) than non-ε4 carriers (15.9 (2.5)) (p=0.0058), but this association was not significant within the AD or non-AD groups. There was no association between the *NEP* polymorphism (the total repeat number or the number of shorter alleles) and *APOE* genotype (data not shown).

DISCUSSION

We showed that the CAA severity was significantly higher in the patients who had an *NEP* polymorphism with up to 40 GT repeats in total than in those with more than 40 repeats. The shorter allele with 19 or 20 repeats was associated with a higher CAA severity.

As the *NEP* polymorphism was not associated with AD in this population,[21] the association between the *NEP* polymorphism and CAA cannot be explained by an association with AD, a strong risk factor of CAA. The association between the *NEP* polymorphism and CAA did not seem to be influenced by the presence of AD or by the status of *APOE* ε4. There was no interaction between the *NEP* polymorphism and *APOE* genotype.

In contrast with our previous study,[21] Streffer *et al* found a positive association between the *NEP* polymorphism and AD (J R Streffer, *et al*, 31st Annual Meeting of Society for Neuroscience, San Diego, 10–15 November, 2001). The 20-repeat

Table 2 Comparison of average CAA counts (mean (SE)) between smaller (40 or less) and larger (41 or more) total repeat numbers of the *NEP* polymorphism

	CAA counts in subjects with		Statistical difference* (p value)
	40 or less total repeats	41 or more total repeats	
Total cases	21.6 (2.7) (n=120)	8.2 (2.8) (n=44)	0.005
AD or non-AD			
AD	33.0 (4.0) (n=59)	20.2 (6.8) (n=16)	0.118
non-AD	10.6 (2.9) (n=61)	1.4 (0.9) (n=28)	0.062
Status of APOE			
ε4 (+)	26.9 (4.8) (n=35)	12.5 (5.9) (n=12)	0.052
ε4 (−)	19.5 (3.2) (n=85)	6.6 (3.2) (n=32)	0.040

*Mann-Whitney U test.

allele was the most frequent in our Japanese population, while the 21-repeat allele was the most frequent in their European population (J R Streffer, *et al*, 31st Annual Meeting of Society for Neuroscience). There may be an ethnic difference in the *NEP* polymorphism. In addition, another recent study with clinical samples from a Japanese population showed lack of association between the *NEP* polymorphism and AD.[26]

As the *NEP* polymorphism is present in the regulatory region upstream of exon 1, this polymorphism may influence the transcription of *NEP* to type 1 mRNA in neurons[17–20]; the dinucleotide repeats may induce conformational changes in the DNA helix that results in changed DNA-protein interactions.[19] The shorter alleles may be associated with decreased levels of the neuronal *NEP* mRNA transcripts, which would result in a decrease of neprilysin activity. In such cases, the decreased neprilysin activity would increase the levels of both Aβ40 and Aβ42 as suggested in the experiment with neprilysin deficient mice.[14] The increased Aβ40 and Aβ42 levels would be linked to a risk of CAA, but the reason why the *NEP* polymorphism was not associated with senile plaques[21] is unknown.

In addition, Comings *et al*[25] reported that low molecular weight alleles for this repeat polymorphism are associated with a low amplitude of P300, a cognitive event related brain potential. They suggested that the lower molecular weight alleles—that is, shorter alleles, are associated with increased levels of enkephalinase (neprilysin) and thus lower CNS enkephalin levels.[25] Their presumption is not consistent with ours concerning Aβ catabolism. It is possible that the association between *NEP* polymorphism with CAA reflects linkage disequilibrium with another polymorphism in *NEP* or a nearby gene. The significance of this polymorphism in the expression of *NEP* and neprilysin activity for proteolysis of Aβ and roles of other *NEP* polymorphisms should be further clarified.

Finally, this is the first report suggesting that a genetic polymorphism of an Aβ degradating enzyme may be associated with risk of CAA. However, this study is limited to a comparatively small sample population obtained from an necropsy series in a geriatric hospital. Our results warrant further study with a larger sample size from populations with various ethnic backgrounds. Although the significance and biological background of this association need to be established, this polymorphism is expected to become a useful biological marker for clinical diagnosis of CAA, and modulation of neprilysin activity would be a therapeutic target.

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